

# Activation of different Wnt/ $\beta$ -catenin signaling components in mammary epithelium induces transdifferentiation and the formation of pilar tumors

Keiko Miyoshi<sup>1,2</sup>, Andrea Rosner<sup>3</sup>, Masahiro Nozawa<sup>1</sup>, Christopher Byrd<sup>1</sup>, Fanta Morgan<sup>1</sup>, Esther Landesman-Bollag<sup>4</sup>, Xin Xu<sup>4,5</sup>, David C Seldin<sup>4</sup>, Emmett V Schmidt<sup>6</sup>, Makato M Taketo<sup>7</sup>, Gertraud W Robinson<sup>1</sup>, Robert D Cardiff<sup>3</sup> and Lothar Hennighausen<sup>\*1</sup>

<sup>1</sup>Laboratory of Genetics and Physiology, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland, MD 20892, USA; <sup>2</sup>Department of Biochemistry, School of Dentistry, the University of Tokushima, Tokushima, Tokushima 770–8504, Japan; <sup>3</sup>Center for Comparative Medicine, University of California, Davis, California, CA 95616, USA; <sup>4</sup>Boston Medical Center, Boston, Massachusetts, MA 02118, USA; <sup>5</sup>McLaughlin Research Institute, Great Falls, Montana, USA; <sup>6</sup>Massachusetts General Hospital, Boston, Massachusetts, MA 02114, USA; <sup>7</sup>Kyoto University Graduate School of Medicine, Kyoto, Japan

**The Wnt/ $\beta$ -catenin signaling pathway controls cell fate and neoplastic transformation. Expression of an endogenous stabilized  $\beta$ -catenin ( $\Delta E3$   $\beta$ -catenin) in mammary epithelium leads to the transdifferentiation into epidermis- and pilar-like structures. Signaling molecules in the canonical Wnt pathway upstream from  $\beta$ -catenin induce glandular tumors but it is not clear whether they also cause squamous transdifferentiation. To address this question we have now investigated mammary epithelium from transgenic mice that express activating molecules of the Wnt pathway: Wnt10b, Int2/Fgf3, CK2 $\alpha$ ,  $\Delta E3$   $\beta$ -catenin, Cyclin D1, and dominant negative (dn) GSK3 $\beta$ . Cytokeratin 5 (CK5), which is expressed in both mammary myoepithelium and epidermis, and the epidermis-specific CK1 and CK6 were used as differentiation markers. Extensive squamous metaplasias and widespread expression of CK1 and CK6 were observed in  $\Delta E3$   $\beta$ -catenin transgenic mammary tissue. Wnt10b and Int2 transgenes also induced squamous metaplasias, but expression of CK1 and CK6 was sporadic. While CK5 expression in Wnt10b transgenic tissue was still confined to the lining cell layer, its expression in Int2 transgenic tissue was completely disorganized. In contrast, cytokeratin expression in CK2 $\alpha$ , dnGSK3 $\beta$  and Cyclin D1 transgenic mammary tissues was similar to that in  $\Delta E3$   $\beta$ -catenin tissue. In support of transdifferentiation, expression of hard keratins specific for hair and nails was observed in pilar tumors. These results demonstrate that the activation of Wnt signaling components in mammary epithelium induces not only glandular tumors but also squamous differentiation, possibly by activating LEF-1, which is expressed in normal mammary epithelium.**

*Oncogene* (2002) 21, 5548–5556. doi:10.1038/sj.onc.1205686

**Keywords:** Wnt signaling;  $\beta$ -catenin activation; Int2/Fgf3; cytokeratin expression; pilar tumor; transdifferentiation

## Introduction

Wnt signaling controls various aspects of development, including cell fate determination and neoplastic transformation (Cadigan and Nusse, 1997; Korinek *et al.*, 1998; Merrill *et al.*, 2001; Miller and Moon, 1996; Polakis, 2000).  $\beta$ -catenin is a central molecule in the canonical Wnt signaling pathway, which is activated by secreted proteins from the Wnt family that bind to receptors from the Frizzled protein family (Bhanot *et al.*, 1996; Tekmal and Keshava, 1997; Wodarz and Nusse, 1998). Upon ligand binding, Dishevelled (dsh) is activated through phosphorylation, possibly by protein kinase CK2, formerly known as casein kinase II (Willert *et al.*, 1997). Activated dsh in turn inactivates glycogen synthase kinase 3 (GSK3). GSK3 phosphorylates serine and threonine residues in the N-terminus of  $\beta$ -catenin and thus prepares it for ubiquitin-mediated degradation (Aberle *et al.*, 1997). Inactivation of GSK3 inhibits N-terminal phosphorylation of  $\beta$ -catenin, such that  $\beta$ -catenin cannot be degraded and translocates into the nucleus (Behrens *et al.*, 1998). In a complex with LEF/TCF transcription factors,  $\beta$ -catenin activates target genes, including Cyclin D1 and *myc* (Behrens *et al.*, 1996; He *et al.*, 1998; Roose *et al.*, 1999; Shtutman *et al.*, 1999; Tetsu and McCormick, 1999).

Over the past decade transgenic mice have been generated, in which the Wnt pathway is activated. Several strategies have been employed to achieve this. First, molecules that activate the Wnt pathway have been expressed ectopically in mammary epithelium under control of the MMTV-LTR. These include Wnt1 (Tsukamoto *et al.*, 1988), Wnt10b (Lane and Leder, 1997), Int2/Fgf3 (Lee *et al.*, 1995; Muller *et al.*, 1990), the  $\alpha$  catalytic subunit of CK2 (Landesman-Bollag *et al.*,

\*Correspondence: L Hennighausen, Laboratory of Genetics and Physiology, NIDDK, NIH, Building 8, Room 101, 8 Center Drive, Bethesda, Maryland, MD 20892-0822, USA;

E-mail: hennighausen@nih.gov

The article is a work of the United States Government and as such must remain in the public domain.

Received 26 February 2002; revised 13 May 2002; accepted 20 May 2002

2001),  $\Delta$ N89  $\beta$ -catenin (Imbert *et al.*, 2001),  $\beta$ -catenin- $\Delta$ N90 (Michaelson and Leder, 2001), and a dominant negative form of GSK3 $\beta$  (dnGSK3 $\beta$ ) capable of activating Wnt signaling (DC Seldin *et al.*, unpublished). In addition, one of the major transcriptional targets of the Wnt pathway, Cyclin D1, has been similarly expressed (Wang *et al.*, 1994). These mice develop mammary tumors with variable latency periods. In another approach, endogenous  $\beta$ -catenin was stabilized through the deletion of exon 3 ( $\Delta$ E3  $\beta$ -catenin, Harada *et al.*, 1999; Miyoshi *et al.*, 2002), which encodes the N-terminal region that is the target of GSK3 $\beta$  phosphorylation. Interestingly, upon the stabilization of  $\beta$ -catenin in mammary epithelium these mice do not develop glandular tumors and the mammary epithelium undergoes transdifferentiation into epidermal- and pilar-like structures (Miyoshi *et al.*, 2002).

The availability of mice, in which different molecules that activate the Wnt pathway are deregulated, provides a unique opportunity to explore their relative contributions to specific developmental and biochemical signaling events. Since the stabilization of  $\beta$ -catenin results in the transdifferentiation of mammary epithelium (Miyoshi *et al.*, 2002), and a quarter of the transgenic mammary tumors induced by canonical Wnt pathway molecules represent squamous metaplasias in pilar tumors (Rosner *et al.*, 2002), we decided to explore whether activation of other components of the Wnt pathway could induce a similar transdifferentiation process. We hypothesized that if these molecules are part of a common signaling pathway, their activation should result in similar phenotypic consequences. To categorize the tumors we analysed the expression of three types of cytokeratins, CK5, CK1 and CK6. CK5 is expressed in both mammary myoepithelium and the basal cell layer in the epidermis (Fuchs and Byrne, 1994; Miyoshi *et al.*, 2002). In contrast, CK1 and CK6 are not expressed in normal mammary epithelium, but they are detected in epidermis (Fuchs and Byrne, 1994; Miyoshi *et al.*, 2002). CK1 is expressed in the spinous layer of the epidermis (Fuchs and Byrne, 1994) and CK6 is a marker for proliferating cells in the epidermis, especially in hair follicles (Vasioukhin *et al.*, 2001; Weiss *et al.*, 1984). Another indicator of transdifferentiation was provided by the antibody AE-13, which recognizes hard keratins specific for hair and nails. Based on this study, the morphology of many lesions in transgenic mice in which the Wnt pathway was activated is reminiscent of hair follicles. Specifically, the expression of follicular keratins connotes a change in mammary epithelial cell differentiation.

## Results

### *Stabilized $\beta$ -catenin induces transdifferentiation of mammary epithelium*

Previously we reported that the deletion of exon 3 of the  $\beta$ -catenin gene ( $\Delta$ E3  $\beta$ -catenin) in mammary

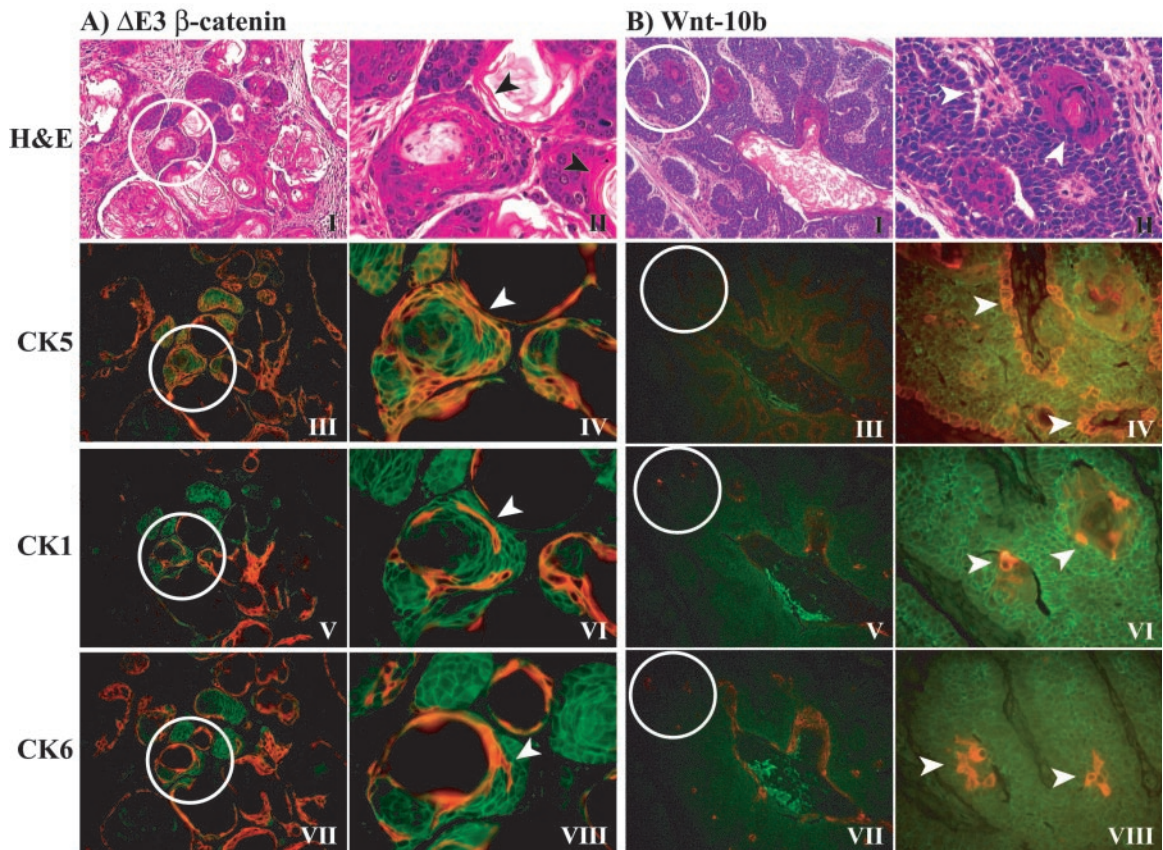
epithelium resulted in the generation of stabilized  $\beta$ -catenin and the transdifferentiation of mammary epithelium into epidermis- and pilar-like structures during the first pregnancy (Miyoshi *et al.*, 2002). Pilar mammary tumors are characterized by abortive hair shafts, ghost cells, confluent swirls of laminar keratin corresponding to former dilated ducts with excessive squamous metaplasia, or combinations of these. Squamous nodules are frequent dysplasias of the mouse mammary gland and have either abortive hair shafts or confluent keratin swirls. In  $\Delta$ E3  $\beta$ -catenin mice, we detected all characteristics of these pilar tumors (Miyoshi *et al.*, 2002) and extensive transdifferentiation was observed in females that had gone through one pregnancy (Figure 1A). Here we analysed six additional mice (Table 1). Keratin deposits or laminae were found in dysplastic squamous nodules (Figure 1A, I and II). The outer layers of the nodules expressed CK5 (Figure 1A, III and IV), and the more central layers expressed CK1 (Figure 1A, V and VI). CK6 expressing cells were frequently located between the CK5 and CK1 layers, and some were interspersed with CK1 expressing cells (Figure 1A, VII and VIII). The acellular keratin lamellae did not stain.

### *Ligand of Wnt signaling pathway: Wnt10b*

Expression of Wnt10b under control of an MMTV-LTR (Wnt10b mice) results in the development of mammary glandular tumors in both males and females (Lane and Leder, 1997). We also detected limited squamous hyperplasias that showed abortive hair shafts (Figure 1B, I and II), ductular structures filled with keratin, which are usually found at the circumference of the tumor. The pattern of cytokeratin expression was investigated in mammary tissue from a Wnt10b transgenic male. CK5 was expressed in the outer cell layer of the tumor mass and on the luminal side (Figure 1B, III and IV). In contrast, the epidermal differentiation markers CK1 and CK6 were expressed sporadically in the center of the glandular tumors (Figure 1B, V-VIII) (Table 1).

### *Kinases of the Wnt signaling pathway: CK2 $\alpha$ and dnGSK3 $\beta$*

Expression of either the  $\alpha$  catalytic subunit of CK2 (CK2 $\alpha$ , Landesman-Bollag *et al.*, 2001), or a dominant negative GSK3 $\beta$  (dnGSK3 $\beta$ , DC Seldin *et al.*, unpublished) under control of an MMTV-LTR induces mammary tumors correlated with increased levels of  $\beta$ -catenin. The dnGSK3 $\beta$  is known to antagonize the wild type kinase and promote Wnt signaling in *Xenopus* embryos. CK2 impinges on the Wnt pathway in multiple places, as it is one of the kinases that phosphorylates dsh during Wnt signaling (Willert *et al.*, 1997) and it is also capable of phosphorylating  $\beta$ -catenin directly, and stabilizing it (Song *et al.*, 2000). Here we have analysed mammary tissue from three multiparous females that contained squamous nodules with epidermal-like keratinization patterns (Figure 2A,



**Figure 1** Histological characteristics of  $\Delta E3$   $\beta$ -catenin (floxed with MMTV-Cre) and MMTV-Wnt10b transgenic mice. (I–II) H&E staining and (III–VIII) immunofluorescence stains with various cytokeratins (CK, red) and  $\beta$ -catenin (green) antibodies. (A)  $\Delta E3$   $\beta$ -catenin mammary tissue. CK5 was expressed in outer layers of the nodules (III and IV). CK1 was expressed in the inner layers of the nodules (V–VI). CK6 staining pattern was observed between CK1 and CK5 expressed cells (VII–VIII) and some overlapped with CK1 (VI–VIII, arrows). II, IV, VI, VIII; 3 $\times$  magnification of the circle area shown in I, III, V, VII, respectively. (B) MMTV-Wnt10b transgenic mammary tissue. CK5 was expressed in the outer and luminal layers of the tumor mass (I–IV). CK1 and CK5 were expressed inside the nodules, but not in the same cells (V–VIII, arrows). CK1 and CK6 expression was sporadic (V–VIII, arrows). II, IV, VI, VIII; 3 $\times$  magnification of the circled areas shown in I, III, V, VII, respectively

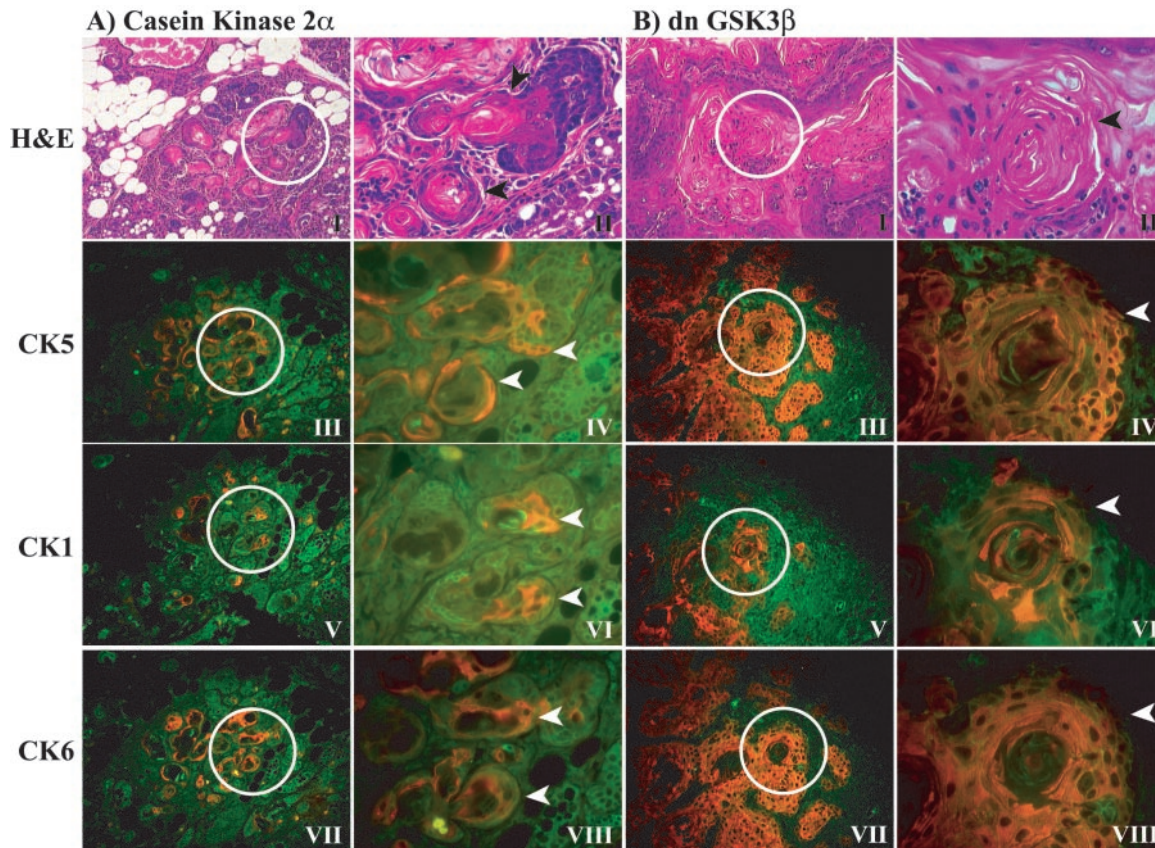
**Table 1** Characterization of pilar tumours in transgenic mice expressing different components of the wnt pathway using cytokeratin expression

Signaling molecule	Published data					This study		
	Tumor type	Latency (months)	# of pilar tumors*	# of analysed pilar tumors	Analysed age	CK5	CK1	CK6
Wnt10b	Adenocarcinomas	2.5–6	1/23	1	4	Lining cells	Some in the center	Some in the center
Int2/FGF3	Hyperplasias, adenocarcinomas	10	7/34	3	8	Not in outer cells, disorganized pattern	A few in the center single cell	A few in the center single cell
CK2 $\alpha$	Adenocarcinomas, squamous metaplasias, spindle cell tumors	median 23	8/29	3	19	Outer cells	Inner cells	Inner cells
dn GSK3 $\beta$			11/36	5	22	Several outer layers	Several inner layers	Between K1 and K5
$\Delta E3$ $\beta$ -catenin (Cre-loxP)	Squamous metaplasias	2	11/11	11	2.5	Outer cell layers	Inner cells or center	Between K1 and K5 or interspersed w/K1
Cyclin D1	Adenocarcinomas, squamous metaplasias	17–21	7/10	5	20.5	Outer cells	Center	Inner cells

\*The number of pilar tumor formed samples/the number of total tumor samples

I and II and Table 1). CK5 was expressed in the outer layers of the nodules (Figure 2A, III and IV). CK1 was expressed in the center (Figure 2A, V and VI) and strong CK6 staining was observed in nodule-like hair follicles (Figure 2A, VII and VIII).

Expression of dnGSK3 $\beta$  resulted in a phenotype similar to that seen in  $\Delta E3$   $\beta$ -catenin mice (compare Figures 1A and 2B, I and II). Squamous metaplasias were found in all five females analysed (Table 1). In addition to the keratinization, we observed extensive



**Figure 2** Histological characteristics of CK2 $\alpha$  and dnGSK3 $\beta$  transgenic mice. (I–II) H&E staining and (III–VIII) immunofluorescence stains with various cytokeratins (CK, red) and  $\beta$ -catenin (green) antibodies. (A) MMTV-CK2 $\alpha$  transgenic mammary tissue. CK5 was expressed in the outer layers of the nodules (I–IV). CK1 was expressed inside the nodules (V–VI, arrows). CK6 staining patterns were similar to hair follicles in wild type skin (VII–VIII, arrows). (B) MMTV-dnGSK3 $\beta$  transgenic mammary tissue. CK5 was highly expressed in several outer layers of nodules (I–IV). CK1 was expressed in the inner layers (V–VI, arrows). CK6 was highly expressed in several layers (VII–VIII, arrows). II, IV, VI, VIII; 3  $\times$  higher magnifications of the circled areas shown in I, III, V, VII, respectively

inflammation in the multiparous female studied (Figure 2B, I and II). Expression of CK5 was not confined to a single cell layer (Figure 2B, III and IV) and CK1 was observed inside of nodules in a laminated pattern (Figure 2B, V and VI). Extensive layers of CK6-positive cells were detected between the CK5- and CK1-positive layers (Figure 2B, VII and VIII). This suggested the presence of areas with extensive cell proliferation. In some cases CK1, 5 and 6 staining was observed within keratinous debris (data not shown).

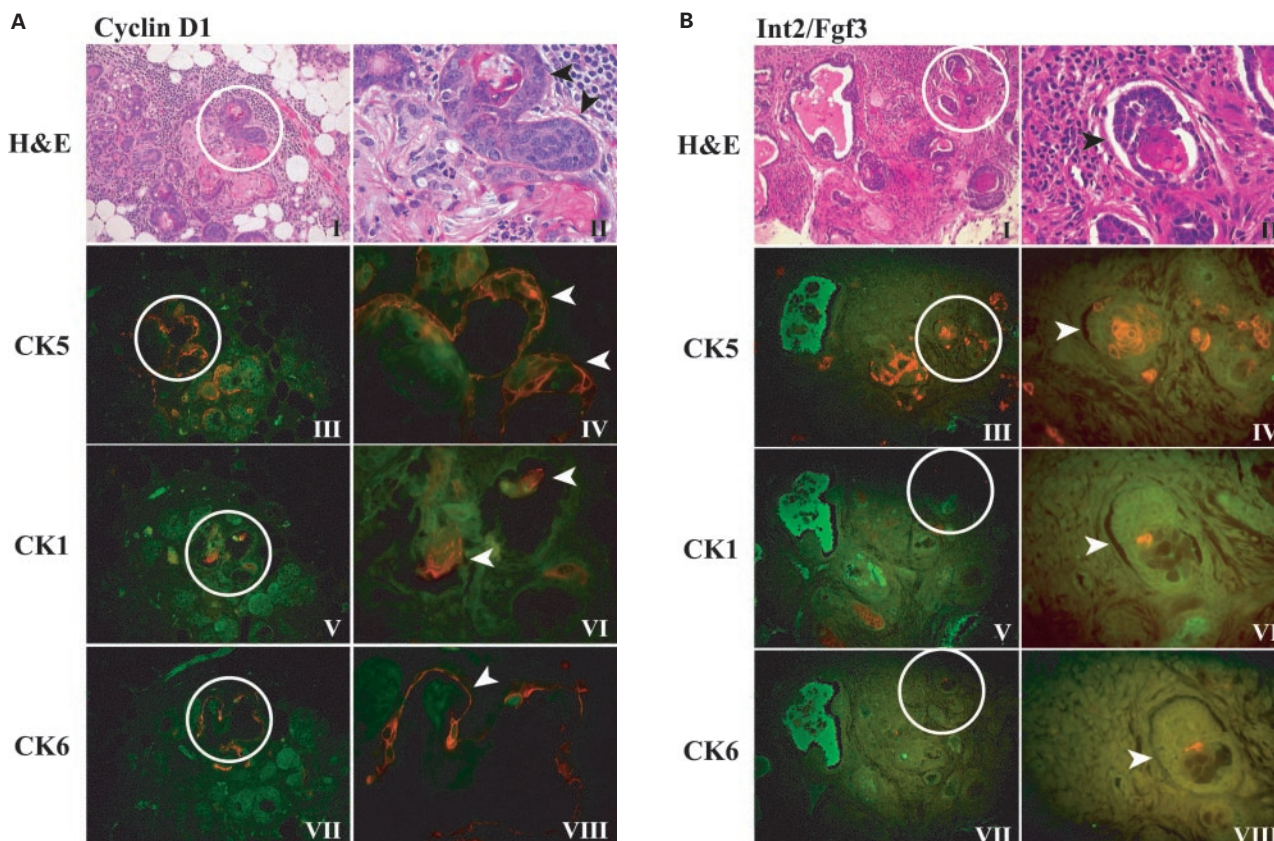
#### A target gene of the Wnt pathway: Cyclin D1

Cyclin D1 is a target gene of the canonical Wnt pathway and MMTV-Cyclin D1 transgenic mice develop hyperplasias and glandular tumors with papillary elements or squamous metaplasias after 12 to 15 months of age (Wang *et al.*, 1994). We analysed a parous female that had developed several pilar dysplasias. Figure 3A shows a mass of cells that surround squamous nodules (panels I and II). These squamous nodules contained abortive hair shafts and keratin debris. The patterns of cytokeratin staining

were similar to that seen in  $\Delta E3$   $\beta$ -catenin mammary tissue. CK5 was expressed in the outer layers of the nodules (Figure 3A, III and IV), and CK1 was found in the center of the ghost cells (Figure 3A, V and VI). CK6 was expressed in the inner cell layers of the tumors next to ghost cells (Figure 3A, VII and VIII). A total of five mice were analysed and exhibited squamous metaplasias (Table 1).

#### A Wnt cooperating gene: *Int2/Fgf3*

The *Int2/Fgf3* gene is frequently activated through MMTV proviral insertions similar to *Int1/Wnt1* (Peters *et al.*, 1984). Transgenic mice that express *Int2/Fgf3* under control of an MMTV-LTR (*Int2/Fgf3* mice) develop ductal hyperplasias, stromal proliferation and mammary tumors within 10 months of age (Lee *et al.*, 1995; Muller *et al.*, 1990). In tissue from three multiparous females we detected pilar type lesions (Figure 3B and Table 1). Ghost cells, i.e. dead cells with fully keratinized cytoplasm, are frequently seen in the centers of concentric keratin swirls, or they fill the lumen of pilar neoplastic ductules. In some cases they are located within



**Figure 3** Histological characteristics of MMTV-Cyclin D1 and Int2/Fgf3 transgenic mice. (I–II) H&E staining and (III–VIII) immunofluorescence stains with various cytokeratins (CK, red) and  $\beta$ -catenin (green) antibodies. (A) MMTV-Cyclin D1 transgenic mammary tissue. CK5 was expressed in outer layers of nodules (I–IV) and CK1 was expressed in the pilar regions (V–VI, arrows). CK6 was expressed in the single inner layers (VII–VIII, arrows) in a pattern similar to that seen in hair follicles, and some CK1 expression was detected. (B) MMTV-Int2/Fgf3 transgenic mammary tissue. Notably, CK5 was not expressed in outer layers but was found inside of the nodules (I–IV, arrows). II, IV, VI, VIII; 3 $\times$  magnifications of the circled areas shown in I, III, V, VII, respectively

dense stroma with inflammatory infiltrates lacking surrounding epithelial cells, as frequently found in human pilomatricomas (Figure 3B, I and II). CK5 was expressed in the center of the nodules, possibly in ghost cells, but was absent in outer layers (Figure 3B, III and IV). CK1 and CK6 were expressed in single cells in the center of the tumor but they did not co-localize. (Figure 3B, V–VIII). As compared with  $\Delta E3$   $\beta$ -catenin tissue (Figure 1A), these cytokeratin expression patterns appeared disorganized and sporadic.

#### Expression of LEF and hair keratins

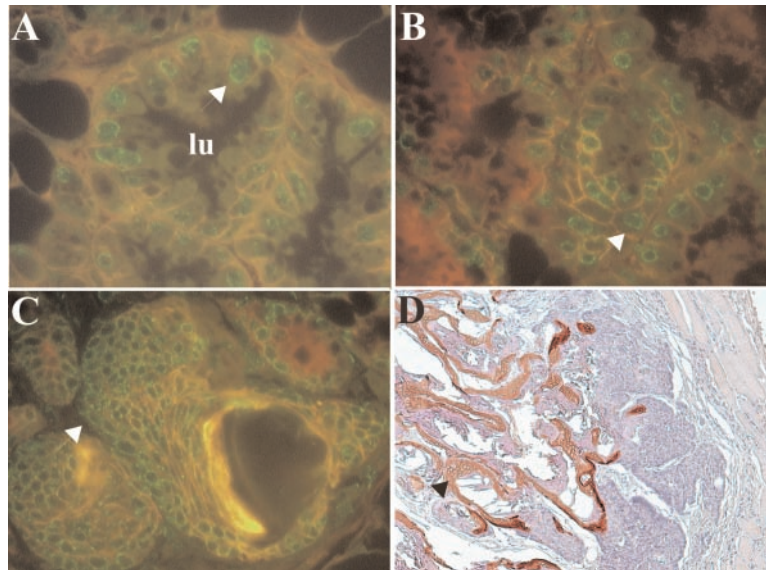
Members of the TCF/LEF HMG domain family are downstream executors of the Wnt signaling pathway and the expression of Tcf-4 has been demonstrated in human mammary epithelium (Barker *et al.*, 1999). In addition, LEF-1 and  $\beta$ -catenin promote hair cell fate (Merrill *et al.*, 2001). We have investigated the expression of LEF-1 in mammary epithelium of  $\beta$ -catenin fl/+; WAP-Cre mice at different stages of transdifferentiation. In areas with maintained normal alveolar structures extensive nuclear LEF-1 staining was observed in the secretory alveolar-like epithelium

(Figure 4A). A different pattern of LEF-1 expression was observed in structures of hyperproliferation and transdifferentiation. Specifically, LEF-1 staining was preferentially perinuclear (Figure 4B,C).

The expression of CK1 is indicative of early epidermal transdifferentiation. To verify the transdifferentiation to pilar cell type, we performed immunohistochemical analyses using the antibody AE13 (Lynch *et al.*, 1986), which recognizes hard keratins specific for hair and nails in all pilar mammary tumors (Figure 4D and Rosner *et al.*, 2002). We have analysed areas of squamous metaplasia that are representative for the pilar mammary tumors frequently observed in the transgenic mice described in this study. Positive staining was limited to ghost cells and to isolated clusters of tumor cells at the peripheral keratinization zone in disrupted neoplastic ductules of CK2 $\alpha$  transgenic mice (Figure 4D).

#### Discussion

Molecules that activate the Wnt signaling pathway have been expressed in mammary epithelium of

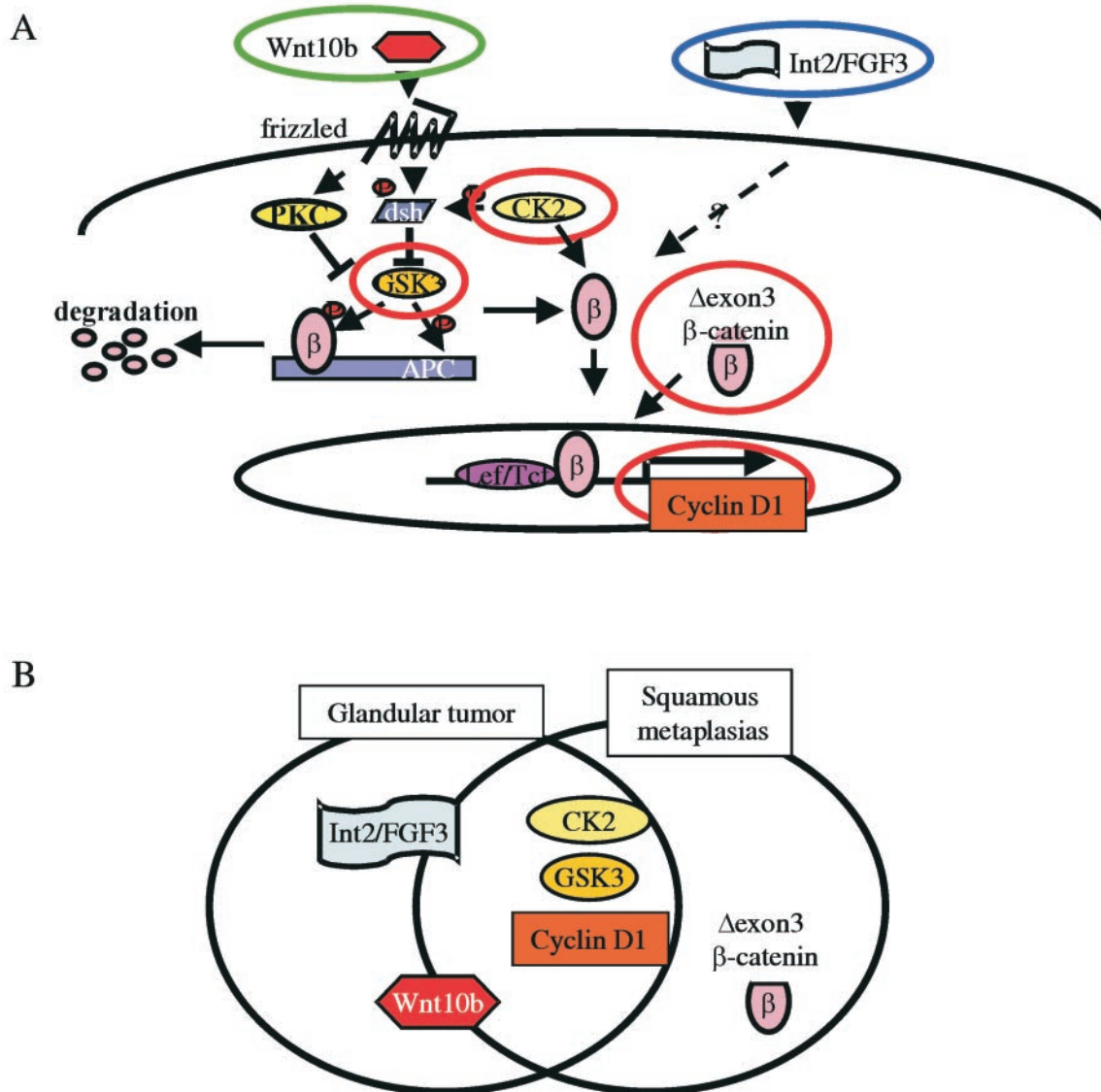


**Figure 4** Expression of LEF-1 in mammary tissue and hard keratin in pilar mammary tumors. (A–C) Mammary tissue from pregnant  $\Delta E3$   $\beta$ -catenin mouse was stained for the presence of LEF-1. Strong nuclear staining was observed in alveolar epithelial cells of normal lobular structures (A). LEF-1 staining was preferentially perinuclear in hyperproliferative areas (B) and structures undergoing squamous transdifferentiation (C). (D) Pilar tumors from a CK2 $\alpha$  mouse were stained with the antibody AE13 that recognizes hard keratins specific for hair and nails. Hard keratin was identified in pilar mammary tumors. Positive staining was limited to ghost cells and to isolated clusters of tumor cells at the peripheral keratinization zone in disrupted neoplastic ductules

transgenic mice. In general these mice develop mammary hyperplasias and glandular tumors (Imbert *et al.*, 2001; Landesman-Bollag *et al.*, 2001; Lane and Leder, 1997; Lee *et al.*, 1995; Michaelson and Leder, 2001; Muller *et al.*, 1990; Tsukamoto *et al.*, 1988; Wang *et al.*, 1994). Beta-catenin is the downstream mediating molecule in the canonical Wnt signaling pathway, and we have demonstrated that the stabilization of endogenous  $\beta$ -catenin (exon 3 deletion) in mammary epithelium induces transdifferentiation into epidermis-like structures (Miyoshi *et al.*, 2002). We hypothesized that if each molecule in the Wnt pathway simply activates  $\beta$ -catenin, we would observe similar types of squamous metaplasias in the different transgenic mice. We tested this hypothesis and characterized mammary tissue of transgenic mice in which this pathway was activated through the expression of Wnt10b, CK2 $\alpha$ , dnGSK3 $\beta$ , Cyclin D1 and  $\Delta E3$   $\beta$ -catenin. We also explored the consequences of Int2/Fgf3 overexpression, a secreted molecule that is not directly linked to the Wnt pathway, but is frequently activated through MMTV insertions (Peters *et al.*, 1984) and cooperates in tumor progression with Wnt-1 (Kwan *et al.*, 1992; Mester *et al.*, 1987; Peters *et al.*, 1986). Transdifferentiation of mammary epithelia into epidermis- and pilar-like structure was observed in all transgenic mice, and we established three distinct cytokeratin staining patterns, which were correlated with specific signaling molecules (Table 1 and Figure 5). The CK1 and CK6 expression patterns suggested that the tumors had undergone epidermal-like transdifferentiation and the CK5 expression patterns permitted us to categorize these tumors. The expression of hard keratins specific for hair and nails further

confirmed the transdifferentiation process. The variability observed in the tumor latency between the different lines of transgenic mice might be due to inherent differences normally seen in transgenic mice, to differences in potency in activation of  $\beta$ -catenin, or to cross-talk with other signaling pathways in which molecules independently are involved. In addition, the MMTV-LTRs used to activate the various transgenes were not identical and in the case of the N-terminally truncated form of  $\beta$ -catenin, the gene was expressed of its endogenous promoter, which most likely has a different regulation than an MMTV-LTR. Furthermore, the transgenic integration sites in combination with the outbred status of the mice are reasons for differences.

Expression of Wnt10b and Int2/Fgf3 induced unique cytokeratin expression patterns and CK2 $\alpha$ ,  $\Delta E3$   $\beta$ -catenin, dnGSK3 $\beta$  and Cyclin D1 mice shared features. In mice that express stabilized  $\beta$ -catenin from its endogenous promoter mammary epithelia transdifferentiate into the epidermis (Miyoshi *et al.*, 2002) and we detected distinct cytokeratin expression patterns in the nodules from the outer to inner layers, similar to that seen in epidermis differentiation from the basal layer to the surface. Although Wnt10b and Int2/Fgf3 induced mainly glandular tumors, we also detected squamous metaplasias. Whereas in Wnt10b tumors CK5 was expressed in an organized pattern in the periphery of the tumor mass, expression in Int2/Fgf3 tumors was disorganized. Specifically, in Int2/Fgf3 tumors, transdifferentiation was initiated in a small number of cells and follicular (or pilar) structures without the establishment of a full set of characteristics. This may be explained by the fact that Int2/Fgf3 can activate a



**Figure 5** Wnt signaling pathway molecules cause squamous metaplasias. (A) Molecules in the signaling pathway that induce the formation of squamous metaplasias. The secreted molecules Wnt (green circle) and Int2/Fgf3 (blue circle) activate distinct pathways and induce glandular tumors and squamous metaplasias. The intracellular signaling molecules GSK3 $\beta$ , CK2 $\alpha$ ,  $\beta$ -catenin and Cyclin D1 are marked with red circles. (B) Distribution of glandular tumors and squamous metaplasias in the different transgenic mice. While  $\Delta$ E3  $\beta$ -catenin induces largely squamous metaplasias, Int2 and Wnt10b induce preferentially glandular tumors. The others produce tumors of mixed phenotypes

variety of signaling pathways (Figure 5A), including MAP kinase, protein kinase B, and protein kinase C (PKC) (Klint and Claesson-Welsh, 1999). Our findings support the notion that Wnt10b also activates other signaling pathways, such as PKC (Cook *et al.*, 1996) to induce tumors (Figure 5A).

Expression of CK2 $\alpha$  and dnGSK3 $\beta$  resulted in similar cytokeratin patterns, suggesting that they enhance  $\beta$ -catenin signaling in the same way as  $\Delta$ E3  $\beta$ -catenin. This is consistent with the roles of these kinases in the regulation of  $\beta$ -catenin and the elevation of  $\beta$ -catenin seen in mammary tumors in these transgenics (Landesman-Bollag *et al.*, 2001; Song *et al.*, 2000). Similarly, the tumor suppressor adenoma-

tous polyposis coli (APC) directly controls  $\beta$ -catenin stability and mice that carry mutant APC alleles develop spontaneous mammary squamous cell carcinomas (Moser *et al.*, 1993; Muller *et al.*, 1990; van der Houven van Oordt *et al.*, 1997). The Cyclin D1 gene is a proposed  $\beta$ -catenin target gene and its overexpression results in squamous metaplasias with a cytokeratin expression pattern similar to that seen in  $\Delta$ E3 $\beta$ -catenin mice. In contrast, the other known target gene of  $\beta$ -catenin in tumors, *c-myc*, induces mainly mammary glandular tumors (Stewart *et al.*, 1984), suggesting that although the canonical Wnt pathway can induce the development of mammary squamous metaplasias, activation of this target gene alone is not sufficient.

The extent of transdifferentiation was dependent on the signaling molecule in the Wnt pathway. While the activation of the downstream molecule  $\beta$ -catenin resulted in the preferential formation of squamous metaplasias, induction of the Wnt pathway with a native ligand Wnt10b resulted in the preferential establishment of glandular tumors (Figure 5B). It should be noted that  $\beta$ -catenin can be activated by Wnt-independent pathways, such as TGF $\beta$  (Smad) and HGF/EGF (Beavon, 2000). Furthermore, our study suggests that signaling pathways independent of  $\beta$ -catenin contribute to the establishment of glandular tumors (Figure 5B). Lastly, cell-specific components determine the fate of a cell after the stabilization of  $\beta$ -catenin. While the stabilization of  $\beta$ -catenin through the deletion of exon 3 in mammary and intestinal epithelium results in the formation of squamous metaplasias (Miyoshi *et al.*, 2002) and polyps (Harada *et al.*, 1999; 2002), respectively, no such foci were observed in liver expressing the stabilized  $\beta$ -catenin (Harada *et al.*, 2002). The molecular basis for this observation could be differential expression of proteins from the TCF/LEF family or the need of additional mutations.

The importance of Wnt signaling for skin differentiation has recently been confirmed by several reports. Deletion of  $\beta$ -catenin in basal cells of the epidermis inhibits the formation of hair follicles (Huelsken *et al.*, 2001). Similarly, expression of  $\Delta$ NLef1, which lacks the  $\beta$ -catenin binding site, in basal cells results in differentiation of hair follicles into squamous epidermal cysts (Niemann *et al.*, 2002). Data using various mutant forms of Tcf3 and Lef1 demonstrate that cell fate determination in the skin is dependent on the relative levels of these  $\beta$ -catenin binding partners (Merrill *et al.*, 2001) and further support our observation of transdifferentiation of mammary epithelial cells by modulating the Wnt signaling pathway.

## Materials and methods

### Transgenic mice

Mammary tissue was harvested from transgenic mice expressing Wnt10b (Lane and Leder, 1997), Int2/Fgf3 (Lee *et al.*, 1995; Muller *et al.*, 1990), CK2 $\alpha$  (Landesman-Bollag *et al.*, 2001), dominant negative GSK3 $\beta$  (dn GSK3 $\beta$ ) (DCS,

unpublished) and Cyclin D1 (Wang *et al.*, 1994) under control of an MMTV-LTR. The  $\Delta$ E3  $\beta$ -catenin gene was under control of its own promoter and deletion was induced by Cre recombinase from a WAP-Cre transgene (Miyoshi *et al.*, 2002). We analysed at least one sample from each transgenic mouse and chose sections that are representative of the histopathology normally observed in these strains. The age of mice that we used here are indicated in Table 1.

### Immunohistochemistry

The fixed samples were embedded in paraffin and sectioned at 5  $\mu$ m. After deparaffinization, dehydration and antigen retrieval by heating in antigen unmasking solution (Vector Laboratories), primary antibodies (cytokeratin 1, 5, or 6, Babco, 1:200;  $\beta$ -catenin, Transduction Labs; 1:100) were applied to the sections. After incubation at 37°C for 1 h, the sections were washed with PBS and incubated with Texas Red-conjugated anti-rabbit (Alexafluor 594) and FITC-conjugated anti-mouse (Alexafluor 488) secondary antibodies (Molecular Probes, 1:400) at 37°C for 30 min. Slides were observed using a Zeiss Axioscop (equipped with filters for FITC, TRITC and FITC:TRITC). Within the mammary tissue adjacent to the tumor, we also analysed normal mammary ducts/alveoli, dysplasias with/without squamous metaplasias, and non-squamous tumors. We captured the images with the strongest staining in squamous metaplasias in each group. Immunohistochemistry with the antibody AE13 was performed as described (Lynch *et al.*, 1986). IHC for hard (hair) keratin was performed on five pilar mammary tumors (transgenes: Int2, Casein kinase 2 $\alpha$ , and dnGSK3 $\beta$ ) using a 1:20 diluted cell culture supernatant with the mouse monoclonal primary antibody AE-13. The Animal Research Kit (DAKO) with peroxidase was used as amplification system according to manufacturer's instructions.

### Acknowledgments

We thank Philip Leder for tissue specimen and T-T Sun for the antibody AE13. Part of this work was supported by grants from the Ministry of Education, Science, Sports and Culture and from the Organization for Pharmaceutical Safety and Research, Japan (MM Taketo), by a grant from NIEHS P01 ES11624 (DC Seldin), by a grant from the German Academic Exchange Service (A Rosner) and by grants 5JB-0014 from the State of California Breast Cancer Research Program (RD Cardiff) and U42 RR14905 from the National Institutes of Health, National Centers for Research Resources (RD Cardiff). The authors wish to acknowledge the excellent technical support provided by Judy E Walls.

## References

- Aberle H, Bauer A, Stappert J, Kispert A and Kemler R. (1997). *EMBO J.*, **16**, 3797–3804.
- Barker N, Huls G, Korinek V and Clevers H. (1999). *Am. J. Pathol.*, **154**, 29–35.
- Beavon IR. (2000). *Eur. J. Cancer*, **36**, 1607–1620.
- Behrens J, Jerchow BA, Wurtele M, Grimm J, Asbrand C, Wirtz R, Kuhl M, Wedlich D and Birchmeier W. (1998). *Science*, **280**, 596–599.
- Behrens J, von Kries JP, Kuhl M, Bruhn L, Wedlich D, Grosschedl R and Birchmeier W. (1996). *Nature*, **382**, 638–642.
- Bhanot P, Brink M, Samos CH, Hsieh JC, Wang Y, Macke JP, Andrew D, Nathans J and Nusse R. (1996). *Nature*, **382**, 225–230.
- Cadigan KM and Nusse R. (1997). *Genes Dev.*, **11**, 3286–3305.
- Cook D, Fry MJ, Hughes K, Sumathipala R, Woodgett JR and Dale TC. (1996). *EMBO J.*, **15**, 4526–4536.
- Fuchs E and Byrne C. (1994). *Curr. Opin. Genet. Dev.*, **4**, 725–736.

- Harada N, Miyoshi H, Murai N, Oshima H, Tamai Y, Oshima M and Taketo MM. (2002). *Cancer Res.*, **62**, 1971–1977.
- Harada N, Tamai Y, Ishikawa T, Sauer B, Takaku K, Oshima M and Taketo MM. (1999). *EMBO J.*, **18**, 5931–5942.
- He TC, Sparks AB, Rago C, Hermeking H, Zawel L, da Costa LT, Morin PJ, Vogelstein B and Kinzler KW. (1998). *Science*, **281**, 1509–1512.
- Huelsken J, Vogel R, Erdmann B, Cotsarelis G and Birchmeier W. (2001). *Cell*, **105**, 533–545.
- Imbert A, Eelkema R, Jordan S, Feiner H and Cowin P. (2001). *J. Cell Biol.*, **153**, 555–568.
- Klint P and Claesson-Welsh L. (1999). *Front Biosci.*, **4**, D165–D177.
- Korinek V, Barker N, Moerer P, van Donselaar E, Huls G, Peters PJ and Clevers H. (1998). *Nat. Genet.*, **19**, 379–383.
- Kwan H, Pecinka V, Tsukamoto A, Parslow TG, Guzman R, Lin TP, Muller WJ, Lee FS, Leder P and Varmus HE. (1992). *Mol. Cell. Biol.*, **12**, 147–154.
- Landesman-Bollag E, Romieu-Mourez R, Song DH, Sonenshein GE, Cardiff RD and Seldin DC. (2001). *Oncogene*, **20**, 3247–3257.
- Lane TF and Leder P. (1997). *Oncogene*, **15**, 2133–2144.
- Lee FS, Lane TF, Kuo A, Shackleford GM and Leder P. (1995). *Proc. Natl. Acad. Sci. USA*, **92**, 2268–2272.
- Lynch MH, O'Guin WM, Hardy C, Mak L and Sun TT. (1986). *J. Cell Biol.*, **103**, 2593–2606.
- Merrill BJ, Gat U, DasGupta R and Fuchs E. (2001). *Genes Dev.*, **15**, 1688–1705.
- Mester J, Wagenaar E, Sluysen M and Nusse R. (1987). *J. Virol.*, **61**, 1073–1078.
- Michaelson JS and Leder P. (2001). *Oncogene*, **20**, 5093–5099.
- Miller JR and Moon RT. (1996). *Genes Dev.*, **10**, 2527–2539.
- Miyoshi K, Shillingford JM, Le Provost F, Gounari F, Bronson R, von Boehmer H, Taketo MM, Cardiff RD, Hennighausen L and Khazaie K. (2002). *Proc. Natl. Acad. Sci. USA*, **99**, 219–224.
- Moser AR, Mattes EM, Dove WF, Lindstrom MJ, Haag JD and Gould MN. (1993). *Proc. Natl. Acad. Sci. USA*, **90**, 8977–8981.
- Muller WJ, Lee FS, Dickson C, Peters G, Pattengale P and Leder P. (1990). *EMBO J.*, **9**, 907–913.
- Niemann C, Owens DM, Huelsken J, Birchmeier W and Watt FM. (2002). *Development*, **129**, 95–109.
- Peters G, Lee AE and Dickson C. (1984). *Nature*, **309**, 273–275.
- Peters G, Lee AE and Dickson C. (1986). *Nature*, **320**, 628–631.
- Polakis P. (2000). *Genes Dev.*, **14**, 1837–1851.
- Roose J, Huls G, van Beest M, Moerer P, van der Horn K, Goldschmeding R, Logtenberg T and Clevers H. (1999). *Science*, **285**, 1923–1926.
- Rosner A, Miyoshi K, Landesman-Bollag E, Xu X, Seldin DC, Moser AR, MacLeod CL, Shyamala G, Gillgrass AE and Cardiff RD. (2002). *Am. J. Pathol.*, in press.
- Shtutman M, Zhurinsky J, Simcha I, Albanese C, D'Amico M, Pestell R and Ben-Ze'ev A. (1999). *Proc. Natl. Acad. Sci. USA*, **96**, 5522–5527.
- Song DH, Sussman DJ and Seldin DC. (2000). *J. Biol. Chem.*, **275**, 23790–23797.
- Stewart TA, Pattengale PK and Leder P. (1984). *Cell*, **38**, 627–637.
- Tekmal RR and Keshava N. (1997). *Front Biosci.*, **2**, D519–D526.
- Tetsu O and McCormick F. (1999). *Nature*, **398**, 422–426.
- Tsukamoto AS, Grosschedl R, Guzman RC, Parslow T and Varmus HE. (1988). *Cell*, **55**, 619–625.
- van der Houven van Oordt CW, Smits R, Williamson SL, Luz A, Khan PM, Fodde R, van der Eb AJ and Breuer ML. (1997). *Carcinogenesis*, **18**, 2197–2203.
- Vasioukhin V, Bauer C, Degenstein L, Wise B and Fuchs E. (2001). *Cell*, **104**, 605–617.
- Wang TC, Cardiff RD, Zukerberg L, Lees E, Arnold A and Schmidt EV. (1994). *Nature*, **369**, 669–671.
- Weiss RA, Eichner R and Sun TT. (1984). *J. Cell Biol.*, **98**, 1397–1406.
- Willert K, Brink M, Wodarz A, Varmus H and Nusse R. (1997). *EMBO J.*, **16**, 3089–3096.
- Wodarz A and Nusse R. (1998). *Annu. Rev. Cell. Dev. Biol.*, **14**, 59–88.